Genetic Diversity Within and Between Hatchery Strains of Japanese Flounder *Paralichthys olivaceus* Assessed by Means of Microsatellite and Mitochondrial DNA Sequencing Analysis[δ]

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Abstract

We assessed genetic divergence within and between hatchery and wild populations of Japanese flounder *Paralichthys olivaceus* by means of microsatellite and mitochondrial DNA (mtDNA) sequencing analysis. Three hundred individuals derived from three hatchery strains and 190 individuals from three wild populations were examined. All 11 microsatellites screened were polymorphic in all samples. Sequences of the mtDNA control region of Japanese flounder were highly variable; of approximately 443 base pairs sequenced, 132 sites were variable among 490 individuals. The number of microsatellite alleles and mtDNA haplotypes, and mtDNA haplotype diversity showed marked reductions in the hatchery strains compared with the wild populations. Both molecular markers yielded high values of *F*-statistics between the hatchery strains, and between the hatchery strains and wild populations. According to a phylogenetic tree topology on the basis of inter-individual genetic relatedness as estimated from microsatellite data, the three hatchery strains were genetically separated, possibly caused by random genetic drift. The DNA markers employed in this study should provide an ideal means for genetic monitoring of Japanese flounder hatchery stocks.

Introduction

The Japanese flounder *Paralichthys olivaceus* is a flatfish species widely distributed throughout coastal areas of Japan and forms an important fishery resource. Recent interest has been directed toward stocking of hatchery -reared fish into natural sea areas to increase the exploitable resource mass. We are concerned with the potential genetic impact of the stocking practice on the wild fish stocks since loss of genetic variability in most hatchery stocks is typical,

δ § *An earlier version of this paper is available in Aquaculture 2002 213: 101-122.*

and this may possibly result in the loss of disease resistance or in the reduction of population's capability to adapt to new environments (Allendorf and Phelps, 1980).

Here, we present an application of two kinds of molecular marker to identical sets of Japanese flounder samples derived from three hatchery stations and three natural sea areas in order to evaluate genetic condition of hatchery strains comparing with that of wild populations. The molecular markers used in this study were eleven microsatellite loci (Sekino and Hara, 2000) and a section of mitochondrial DNA (mtDNA). We also aimed to document potential uses of the molecular markers for further genetic monitoring of Japanese flounder hatchery stocks.

Materials and Methods

Fish Samples

Wild Japanese flounder samples were collected from the Japan Sea in Hokkaido Prefecture (HKD, 50 individuals), from Tottori Prefecture (TTR, 69 individuals) and from the Pacific Ocean in Chiba Prefecture (CHB, 72 individuals). Hatchery fish were provided from a hatchery station in Hokkaido Prefecture (HH, 100 individuals), in Tottori Prefecture (HT, 100 individuals) and in Miyagi Prefecture (HM, 100 individuals). Figure 1 shows the geographical positions of the samples examined. All individuals in the HH strain were F_1 offspring from approximately 110 wild flounder (50 females and 60 males) sampled from off Hokkaido Prefecture.

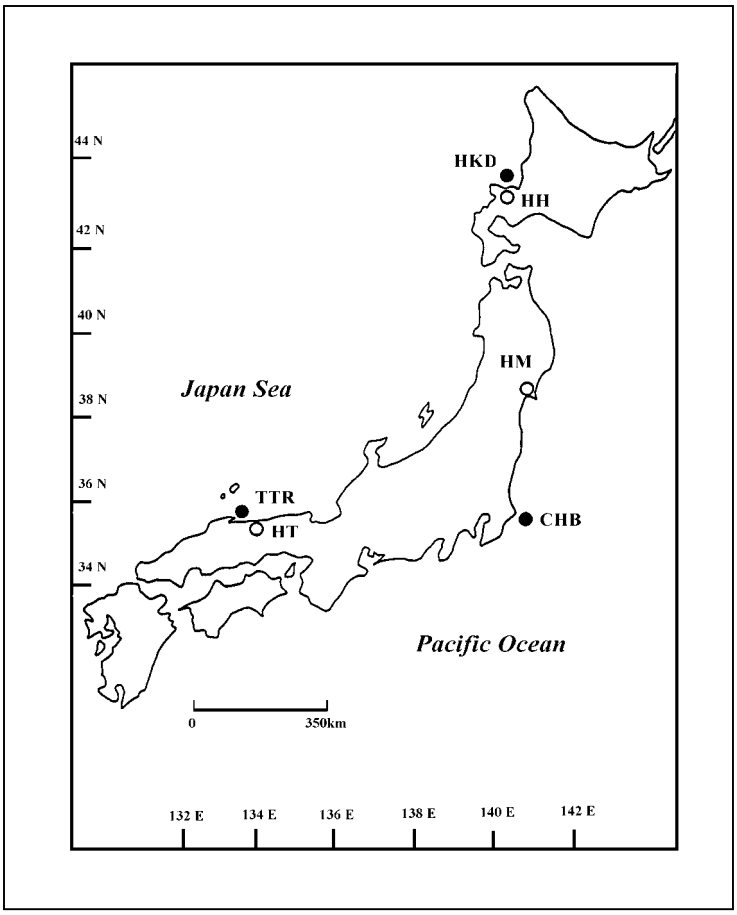

Figure 1. Geographic positions of six Japanese flounder samples. The black dots indicate three sample sites of wild populations; the open dots indicate the location of hatchery stations

The HT strain was founded using approximately 300 individuals. One hundred individuals (50 females and 50 males) were mated in each of three aquarium tanks and the offspring sampled from each tank were communally reared in one tank. The candidate parents were both wild flounder sampled from Tottori Prefecture and brood-stock hatchery-reared potentially over several generations. There are no available records showing the number of siblings used for this strain. The HM population originated from approximately 60 wild flounder (30 females and 30 males) sampled from Miyagi Prefecture, comprised of F_1 offspring of the wild captives. Genetic information on the candidate parents from the three hatchery strains was not available.

Polymorphism Screening

Eleven microsatellites, *Po1*, *Po13*, *Po25A*, *Po26*, *Po33*, *Po35*, *Po42*, *Po48*, *Po52*, *Po56*, and *Po91* were screened in this study. The PCR amplification condition for each locus is available in Sekino and Hara (2000). Mendelian inheritability for each locus was verified in our previous study (Sekino and Hara, 2001). Microsatellite polymorphisms were screened using an ALF express automated DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden).

According to a complete nucleotide sequence of Japanese flounder mtDNA genome (Saitoh *et al.,* 2000, GenBank accession AB028664) we designed one set of PCR primer pair to amplify approximately 480 base pair (bp) segments flanking the $tRNA^{Pro}$ gene and the left domain of the control region: 2 primers were placed in the $tRNA^{Thr}$ gene (forward primer: 5'-GTT AGA GCG CCA GTC TTG TA-3') and the middle of the control region (reverse primer: 5'-CCT GAA GTA GGA ACC AAA TGC-3'). The PCR amplification was carried out in a 10 μl reaction mixture, which included 10 pmols of each primer, 100 μM of dNTPs, 10 mM Tris-HMl (pH8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 units of DNA polymerase (ExTaq^{TM}, Takara, Shiga, Japan). Approximately 50 ng of template DNA. PCR cycles were as follows: 3 min at 95, 30 cycles of 15 s at 95 , 30 s at 57 , and 30 s at 72 , and final elongation for 5 min at 72 . Sequencing analysis for the PCR amplification products was performed using an ABI 373A stretch DNA sequencer (Applied Biosystems, Foster City, CA USA). Sequences were determined from both directions.

Statistical Analysis

Microsatellite allele frequencies and expected heterozygosity (*He*) of each population at each locus were estimated using an ARLEQUIN version 1.1 software package (Schneider *et al.,* 1997). The observed heterozygosity (*Ho*) was calculated directly from the observed genotypes. We used the ARLEQUIN program to estimate an overall inbreeding coefficient (F_{IS} ; Weir and Cockerham, 1984).

Sequence alignment of mtDNA sequence data was performed using a sequence editor DNASIS software package (HITACHI, Tokyo, Japan). The number of variable sites, haplotype frequency distributions and haplotype diversity were calculated using the ARLEQUIN program. The haplotype diversity was based on the formula $h = (1 - \Sigma x_i^2)\, n / (n - 1)$, where x_i is the frequency of a haplotype and n is the sample size (Nei and Tajima, 1981).

Overall *F*-statistics (Weir and Cockerham, 1984) was estimated based on both microsatellites (F_{ST}) and mtDNA sequences (Φ_{ST}) using the ARLEQUIN program. Genetic relationships between individuals within and between hatchery strains were estimated. First, we defined the term "allele frequency in an individual" as follows: if individual *X* had genotype *AA* at locus *L*, the frequency of allele *A* at that locus in the individual *X* was defined as *A* =1.0; if individual *X* had genotype *AB* at that locus, the frequency of allele *A* and *B* in the individual *X* was defined as *A* = 0.5 and *B* = 0.5. Then, we estimated inter-individual genetic similarity according to a formula $I = \Sigma X_i Y_i / (\Sigma X_i^2 \Sigma Y_i^2)^{1/2}$, where X_i and Y_i is the frequency of *i* th allele for each locus in the individual *X* and *Y*, respectively. We calculated the *I* values for all possible pairwise combinations of individuals for all loci, and then a pairwise genetic distance measure was calculated as $D = (1 - I_k)$, where I_k is the average of the *I* values calculated for each locus. A phylogenetic tree topology based on the distance measure was constructed according to a neighbor-joining method (Saitou and Nei, 1987). This analysis was performed using 80 individuals per hatchery strain, that is, a total of 240 individuals were used for this analysis.

Results

Table 1 summarizes the microsatellite variabilities. The variability estimated for the hatchery strains is characterized as substantial reductions of the number of alleles per locus (hatchery strains*:* 5.9-10.7; wild populations: 15.3-18.2). Overall expected heterozygosity (*He*) ranged from 0.59 to 0.71 in the 3 hatchery strains, and from 0.75 to 0.76 in the 3 wild populations.

Table 1. Microsatellite variabilities in six Japanese flounder samples.

	Hatchery strains			Wild populations		
	HH	**HT**	**HM**	**HKD**	**TTR**	**CHB**
Number of loci examined	11	11	11	11	11	11
Sample size	100	100	100	50	69	72
Hardy-Weinberg disequilibrium [*1]	8	4	3	0	0	0
Number of alleles per locus (*A*)	10.7	5.9	10.0	15.3	17.5	18.2
Overall observed heterozygosity (*Ho*)	0.72	0.57	0.72	0.77	0.78	0.75
Overall expected heterozygosity (*He*)	0.71	0.59	0.70	0.75	0.76	0.75
Overall F_{IS}	-0.021	0.018	-0.037	-0.023	-0.028	-0.001
P [*2]	0.86	0.20	0.98	0.88	0.95	0.55

[*1] *Number of loci that showed significant departure from Hardy-Weinberg's equilibrium. The probability was tested analogously to Fisher's exact test in the Markov-chain method, with initial K of sequential Bonferroni correction (Rice, 1989) K=11 (P<0.005)*

[*2] *Probability value associated with the* $F_{IS.}$

Sequences containing the $tRNA^{Pro}$ gene (71bp) and the left domain of the mtDNA control region turned out to be highly variable: of approximately 443 nucleotides, which we unambiguously determined for a total of 490 individuals, there were 132 variable sites consisting of 149 base-substitutions with 5 single base pair insertion/deletion (Fig. 2). Accordingly, a total of 179 haplotypes were identified in the 490 individuals. Haplotypic variabilities estimated for the 6 samples are summarized in Table 2. The 3 hatchery strains did not share any common haplotypes with each other. All hatchery strains had a lower haplotype diversity (*h*=0.692-0.798) than the wild populations (*h*=0.998). There were marked reductions as regards both the number of mtDNA haplotypes and haplotype diversity even in the first-generation hatchery strains (i.e., the HH and HM strains).

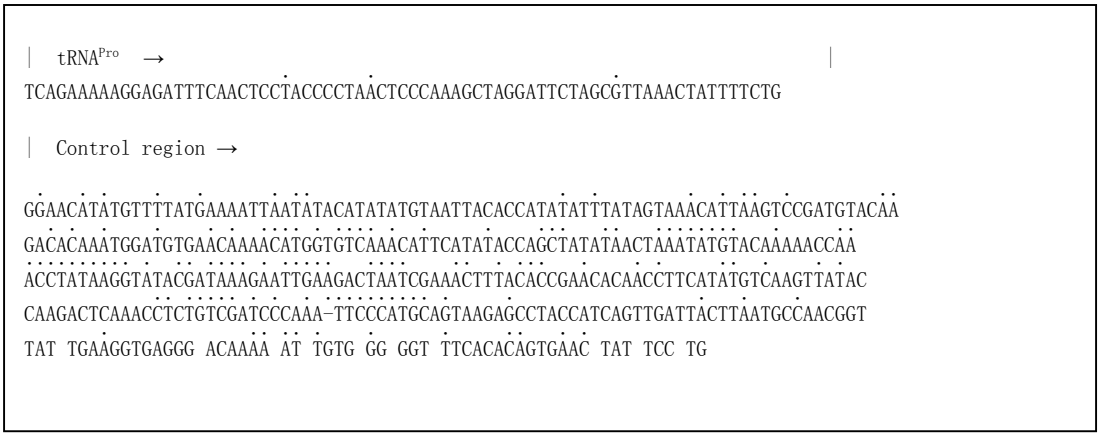

Figure 2. Sequence of the tRNAPro genes and the left domain of the control region of mtDNA of Japanese flounder. Dots indicate variable sites found in at least one haplotype; dashes indicate the single nucleotide deletion/insertion.

Table 2. Mitochondrial DNA variabilities in 6 Japanese flounder samples.

	Hatchery strains			**Wild populations**		
	HH	**HT**	**HM**	**HKD**	**TTR**	**CHB**
Sample size	100	100	100	50	69	71
Number of variable sites	43	29	37	76	87	103
Number of haplotypes	14	4	7	48	65	66
Haplotype diversity *(h)**	0.798	0.692	0.793	0.998	0.998	0.998

Table 3 shows overall *F*-statistics estimated based on both microsatellites (F_{ST}) and mtDNA sequences (Φ_{ST}). A high level of sample-differentiation with statistically significant F_{ST} (Φ_{ST}) was estimated among the hatchery strains (P<0.001). We compared each hatchery strain with the geographically proximal wild population (i.e., HH *vs* HKD, HT *vs* TTR, and HM *vs* CHB). The F_{ST} and Φ_{ST} values estimated for all of the 3 combinations were significantly different from zero (P<0.001).

Table 3. Estimates of F_{ST} (Φ_{ST}) value based on microsatellites and mtDNA sequences.

	Microsatellites		MtDNA	
Sample Combinations	F_{ST}	P^{*1}	Φ_{ST}	P
Among hatchery strains	0.088**	0.000	0.187**	0.000
Among wild populations	0.004*	0.003	0.005	0.134
HH *vs.* HKD	0.026**	0.000	0.084**	0.000
HT *vs.* TTR	0.086**	0.000	0.150**	0.000
HM *vs.* CHB	0.034**	0.000	0.079**	0.000

*[*1] Probability value associated with the F_{ST} (Φ_{ST}) is shown. The F_{ST} (Φ_{ST}) values significantly greater than zero, based on random allelic permutation testing, are noted by adding *=P<0.005 and **=P<0.001.*

According to the NJ tree topology constructed on the basis of the inter-individual genetic similarity (Fig. 3), almost all of individuals derived from each hatchery strain were closely combined, excepting 3 instances as 3 individuals derived from the HH strain were closely clustered with individuals from the HM strain.

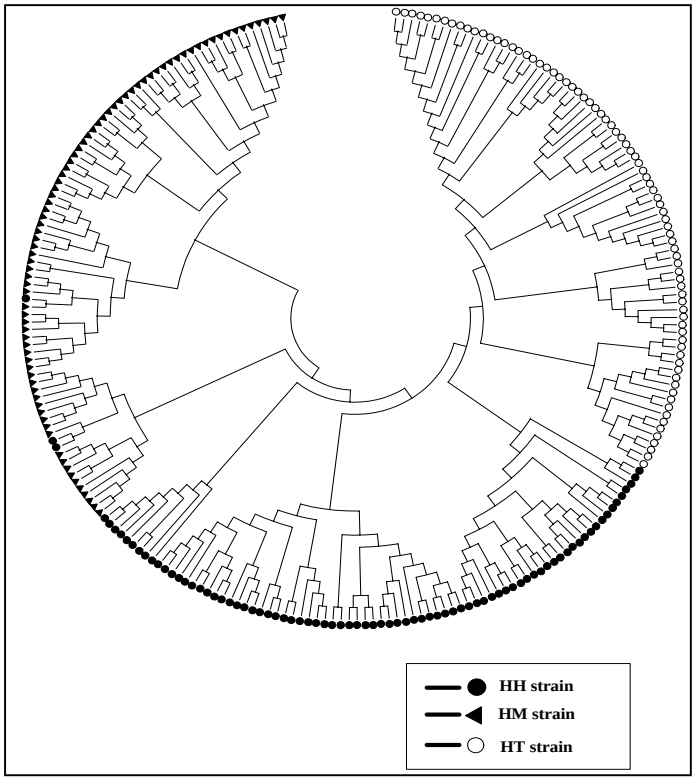

Figure 3. NJ-tree topology, as determined by midpoint rooting, that shows the genetic relationships among 240 individuals randomly chosen from three hatchery strains. Genetic similarity between individuals was calculated on the basis of 11 microsatellite genotypes by using a formula analogous to the genetic identity index between populations (Nei, 1987).

Discussion

A substantial reduction of the number of alleles per locus observed in all of the 3 hatchery strains suggests that each hatchery strain was bottlenecked (Table 1). This is due most likely to the small a number of effective parents when each population was founded. Overall expected heterozygosity (*He*), however, did not show pronounced differences between the hatchery strains and wild populations excepting 1 instance: a significant reduction of the *He* value was observed in the HT strain (see below). These results are not surprising since an estimate of heterozygosity could be inflated if a hatchery strain was founded using heterozygous parents. We therefore consider that the *He* value should not necessarily be useful to evaluate a potential reduction of genetic variation so far as in a first-generation hatchery strain. As an effect of bottlenecking and inbreeding increases, a possibility of significant losses of heterozygous individuals however should increase. It is plausible to consider that the significant reductions of *He* value detected in the HT strain would be caused by population bottleneck together with occurrences of inbreeding events when this strain was founded. This is because the HT strain was founded using both wild caught flounder and brood-stock maintained in this hatchery station, the level of inbreeding however seems not to be high since homozygote excess was not evident in this strain (*Ho*/*He*=0.97), and since the F_{IS} value estimated for this strain was indeed higher compared with other samples but not significant (F_{IS}=0.018, *P*=0.20).

Small a number of mtDNA haplotypes identified in the hatchery strains (4-14 haplotypes) was in contrast to large a number of haplotypes identified in the wild populations (48-66 haplotypes) (Table 2). Considering the fact that the large number of haplotypes were observed in the wild populations (160 haplotypes in 190 individuals), and that the HH and HM strains were first-generation of wild caught flounder, it seems reasonable to assume that the number of haplotypes detected in the HH strain (14 haplotypes) and the HM strain (7 haplotypes) represents the actual number of female parents in each strain. Given that the HH strain was founded using approximately 50 females and the HM strains using 30 females, it can be concluded that only 25% of the candidate female parents for both strains (HH strain: 14/50; HM strain: 7/30) were effective to found each strain.

High F_{ST} and Φ_{ST} values estimated for between the hatchery strains, and between the hatchery strains and wild populations (Table 3), do indicate that there is pronounced genetic differentiation between these samples, possibly caused by random genetic drift. The NJ tree topology showing inter-individual genetic relationships seems to be consistent with genetic drift occurred in hatchery strains as well.

The present study demonstrated that the simultaneous use of the 11 microsatellite loci and the sequences of the mtDNA control region is a powerful approach to monitor genetic condition in Japanese flounder hatchery strains. It should be noted that further extensive stocking practice without any consideration of genetic impact on wild populations might possibly result in irredeemable losses of alleles/haplotypes in natural stocks. The only way to minimize the genetic impacts is to improve the genetic management for all hatchery strains by means of monitoring the genetic variability, estimating precise effective population size. A parentage analysis should provide the most efficient means for this purpose, and we suggest that both microsatellite and mtDNA sequencing technique have the potential to be of great use for this approach.

Acknowledgments

We wish to express our gratitude to Dr. K. Saito, Tohoku National Fisheries Research Institute, for the technical advice on the mtDNA sequencing analysis. Thanks are due to the staff of the Tottori Prefectural Fisheries Station, Hokkaido Central Fisheries Experimental Station, and Miyagi Prefectural Fish Farming Station, for collecting the fish samples.

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